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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371

SPO-112

U.S. APPLICATION NO. (if known, see 37 CFR 1.51)

09/762842

INTERNATIONAL APPLICATION NO.

PCT/JP99/04332

INTERNATIONAL FILING DATE

10 August 1999

PRIORITY DATE CLAIMED

28 March 1999

10 August 1998

TITLE OF INVENTION High Level Secretary Expression System Of Intact  
MK Family ProteinAPPLICANT(S) FOR DO/EO/US Akira Murasugi, Yukio Asami,  
Isao Kido, Hidesh Kumai

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
  - b. ☒ has been communicated by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(3)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19(35 U.S.C. 371(c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (unsigned)
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

## Items 11 to 16 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.  
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: Submission of Sequence Listing Under 37 CFR 1.821-1.825

U.S. APPLICATION NO. (if known) <b>09/762842</b>		INTERNATIONAL APPLICATION NO. <b>PCT/JP99/04332</b>		ATTORNEY'S DOCKET NUMBER <b>SPO-112</b>	
17. <input checked="" type="checkbox"/> The following fees are submitted: <b>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):</b> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... <b>\$1000.00</b> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO. .... <b>\$860.00</b> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <b>\$710.00</b> International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <b>\$690.00</b> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... <b>\$100.00</b> <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				<b>CALCULATIONS</b> PTO USE ONLY	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	14 - 20 =	0	X \$18.00	\$ .00	
Independent claims	3 - 3 =	0	X \$80.00	\$ .00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$270.00	\$ .00	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$ 860.00	
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$	
<b>SUBTOTAL =</b>				\$	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
<b>TOTAL NATIONAL FEE =</b>				\$ 860.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property				\$	
<b>TOTAL FEES ENCLOSED =</b>				\$ 860.00	
				Amount to be refunded:	\$
				charged:	\$

- a. ☐ A check in the amount of \$\_\_\_\_\_ to cover the above fees is enclosed.
- b. ☒ Please charge my Deposit Account No. 19-0065 in the amount of \$ 860.00 to cover the above fees.  
A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any  
overpayment to Deposit Account No. 19-0065. A duplicate copy of this sheet is enclosed.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

Mr. David R. Saliwanchik  
 Saliwanchik, Lloyd & Saliwanchik  
 A Professional Association  
 2421 N.W. 41st Street, Suite A-1  
 Gainesville, FL 32606-6669

*David Saliwanchik*  
 \_\_\_\_\_  
 SIGNATURE  
 David R. Saliwanchik  
 \_\_\_\_\_  
 NAME  
 31,794  
 \_\_\_\_\_  
 REGISTRATION NUMBER

February 12, 2001

Patent Application  
Docket No. SPO-112

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) : Akira Murasugi, Yukio Asami, Isao Kido, Hideshi Kumai  
Docket No. : SPO-112  
For : High Level Secretory Expression System Of Intact MK Family Protein

BOX PCT  
Assistant Commissioner for Patents  
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Please amend the above-identified patent application as follows:

In the Sequence Listing

Please replace existing pages 1-5 of the Sequence Listing with new pages 1-6 of the Sequence Listing.

In the Claims

Please amend the following claims:

Claim 5 (amended):

A transformant comprising methylotrophic yeast transformed with [the vector according to any one of claims 1 to 4] a vector for secretory expression of an intact MK family protein, said vector comprising a gene encoding a mature MK family protein ligated to a signal sequence of  $\alpha 1$  factor derived from *Saccharomyces cerevisiae*.

Claim 6 (amended):

The transformant according to claim 5, wherein said transformant is pPIC9DP-hMK/SMD1168, said MK family protein is MK protein [vector is the one according to claim 3], and said methylotrophic yeast is strain SMD1168.

Claim 7 (amended):

The transformant according to claim 5, wherein said transformant is pPIC9-hPTN/GS115, said MK family protein is PTN protein [vector is the one according to claim 4], and said methylotrophic yeast is strain GS115.

Claim 8 (amended):

A method for producing an intact MK family protein, said method comprising culturing [the transformant according to any one of claims 5 to 7] a transformant comprising methylotrophic yeast transformed with a vector for secretory expression of an intact MK family protein, said vector comprising a gene encoding a mature MK family protein ligated to a signal sequence of  $\alpha 1$  factor derived from *Saccharomyces cerevisiae* and recovering secretory expression products.

Claim 9 (amended):

The method according to claim 8, said method comprising:

- (a) culturing [the transformant according to claim 6] a transformant comprising methylotrophic yeast transformed with a vector for secretory expression of an intact MK family protein, said vector comprising a gene encoding a mature MK family protein ligated to a signal sequence of  $\alpha 1$  factor derived from *Saccharomyces cerevisiae*, wherein said transformant is pPIC9DP-hMK/SMD1168, said MK family protein is MK protein, and said methylotrophic yeast is strain SMD1168,

- (b) inducing the expression of MK protein under the conditions of 20°C and pH 3 after [the] proliferation at pH 4, and
- (c) recovering secretory expression products.

Please add the following new claims:

1           10. The transformant, according to claim 5, wherein said vector comprises  
2           (a) a promoter sequence of a methanol-inducible alcohol oxidase gene (AOX1)  
3           derived from *Pichia pastoris*,  
4           (b) a signal sequence of  $\alpha 1$  factor derived from *Saccharomyces cerevisiae*,  
5           (c) a gene encoding a mature MK family protein, wherein said gene is ligated to (b),  
6           (d) a transcription termination sequence of a methanol-inducible alcohol oxidase gene  
7           (AOX1) derived from *Pichia pastoris*,  
8           (e) a selection marker gene functioning in *Escherichia coli* and methylotrophic yeast,  
9           (f) a replication origin functioning in *Escherichia coli*, and  
10           (g) 5' AOX1 and 3' AOX1 for the site-specific homologous recombination to a  
11           methylotrophic yeast chromosomal DNA.

1           11. The transformant, according to claim 5, wherein said MK family protein is MK  
2           protein.

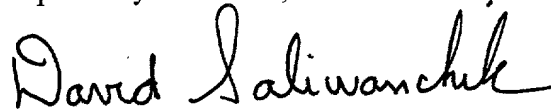
1           12. The transformant, according to claim 5, wherein said MK family protein is PTN  
2           protein.

1           13. The method, according to claim 8, wherein said transformant is  
2 pPIC9DP-hMK/SMD1168, said MK family protein is MK protein, and said methylotrophic  
3 yeast is strain SMD1168.

1           14. The method, according to claim 8, wherein said transformant is  
2 pPIC9-hPTN/GS115, said MK family protein is PTN protein, and said methylotrophic yeast  
3 is strain GS115.

The Commissioner is hereby authorized to charge any fees under 37 CFR 1.16 or  
1.17 as required by this paper to Deposit Account 19-0065.

Respectfully submitted,



David R. Saliwanchik

Patent Attorney

Registration No. 31,794

Phone No.: 352-375-8100

Address : 2421 N.W. 41st Street

Suite A-1

Gainesville, FL 32606

DRS/la

## DESCRIPTION

## HIGH LEVEL SECRETORY EXPRESSION SYSTEM OF INTACT MK FAMILY PROTEIN

5 Technical Field

The present invention relates to a high level secretory expression system of an intact MK family protein by recombinant DNA techniques using methylotrophic yeast as the host.

10 Background Art

MK is a growth factor discovered as the retinoic acid responsive gene product, and is a polypeptide that is rich in basic amino acids and cysteine and that has the molecular weight 13 kDa (Kadomatsu, K. et al.: Biochem. Biophys. Res. Commun., 151: 1312-1318, 1988; Tomomura, M. et al.: J. Biol. Chem., 265: 10765-10770, 1990). MK has 45% sequence homology to another heparin-binding protein referred to as pleiotrophin (PTN) or heparin-binding growth associated molecule (HB-GAM).

MK and PTN have common activities such as the neurotrophic factor activity (Li, Y.-S. et al.: Science, 250: 1690-1694, 1990; Merenmies, J. & Rauvala, H.: J. Biol. Chem., 265: 16721-16924, 1990; Muramatsu, H. et al.: Dev. Biol., 110: 284-296, 1985), the enhancement of fibrinolytic system (Kojima, S. et al.: J. Biol. Chem., 270: 9590-9596, 1995), proliferation of various cells, transformation of NIH3T3 cells (Kadomatsu, K. et al.: Brit. J. Cancer, 75: 354-359, 1997; Yokota, C. et al.: J. Biochem., 123: 339-346, 1998), and angiogenesis.

Thus, these MK family proteins are expected to be useful as drugs, and the development of high level expression system of these proteins has been strongly demanded. Natural MK and PTN proteins are not glycosylated. Therefore, the high level expression of these proteins with no glycosylation by recombinant DNA techniques, would be extremely useful not only for production of the proteins as drugs but also for structural and functional analyses of the proteins. In this invention, an unglycosylated MK family protein is referred to as an intact MK family protein. Herein, an MK family protein means a protein comprising at least the amino acid sequence of a mature

protein of MK, PTN, or their functionally equivalent mutants. In addition, unglycosylated MK and PTN are herein referred to as intact MK and intact PTN, respectively.

As the expression system for an MK family protein, the methanol-metabolizing yeast (referred to as "methyilotrophic yeast" hereafter) is thought to be appropriate. In general, from the viewpoint that yeast is a unicellular eucaryote, abundant in molecular biological information, safe for humans, and easily cultured, it has been used as the host cell in the production of useful proteins by recombinant DNA techniques. In the secretory expression system of yeast, in particular, since the expressed protein is released to the outside of cells, the continuous culturing can be carried out with the expectation of a great deal increase in the production. Furthermore, since the labor to disrupt cells is saved, protein purification becomes easy.

In view of these facts, the present inventors have attempted to establish a high level secretory expression system of MK proteins using methyilotrophic yeast, *Pichia pastoris*, as the host. An expression system of foreign genes by *Pichia pastoris* has been developed and reported in the production of hepatitis type B vaccine and a high-level secretory expression of invertase (Cregg, J. M. et al.: Bio/Technology, 11: 905-910, 1993). However, in the case where the secretion signal unique to MK protein is used for its expression, the expression level thereof is low (30-50 mg/L), and, furthermore, a majority of MK proteins thus expressed are bound to sugars originating in yeast which are different from those attached in animal cells. That is, the content of intact MK protein is extremely low. Use of MK proteins having sugars derived from yeast as drugs poses problems of antigenicity. Therefore, it becomes necessary to isolate and purify intact MK protein from expression products. However, it is highly difficult to isolate and purify proteins that have a common amino acid sequence but differ only in the attached sugars.

To increase an expression level of intact MK protein, the present inventors produced a large number of expression host strains in which the copy number of the expression cassette was increased, and allowed them to express the protein. They obtained yeast strains that produce



an about 2-fold higher expression level than that obtained in conventional strains in the fermentor culture. However, they failed to acquire strains that highly express intact MK protein, indicating that an increase in the copy number is not directly related to the expression of MK protein.

#### Disclosure of the Invention

An object of the present invention is to establish a high level secretory expression system of an intact MK family protein using methylotrophic yeast as a host.

To solve the above-described problems, the present inventors have actively studied and, as a result, constructed an expression vector for an MK family protein by ligating cDNA encoding a mature MK family protein immediately downstream of the prepro-sequence of  $\alpha 1$  factor gene of *Saccharomyces cerevisiae* under the regulation of an alcohol oxidase promoter of methylotrophic yeast, transformed methylotrophic yeast using the vector, and found that the transformant thus obtained expressed a large amount of the active form of an intact MK family protein and secreted it into the culture medium. Furthermore, the present inventors have elucidated that a combination of the signal sequence of  $\alpha 1$  factor with a gene encoding a mature MK family protein is important for the expression of an intact MK family protein, completing this invention.

Specifically, the present invention relates to the following vectors and method for producing an intact MK family protein, the method comprising culturing transformants transformed with these vectors and recovering an intact MK family protein as a secretory expression product.

- (1) A vector for secretory expression of an intact MK family protein in methylotrophic yeast, said vector comprising a gene encoding a mature MK family protein ligated to a signal sequence of  $\alpha 1$  factor derived from *Saccharomyces cerevisiae*.
- (2) The vector according to (1) comprising components (a) to (g) below:
  - (a) a promoter sequence of a methanol-inducible alcohol oxidase gene (AOX1) derived from *Pichia pastoris*,
  - (b) a signal sequence of  $\alpha 1$  factor derived from *Saccharomyces*

*cerevisiae*,

- (c) a gene encoding a mature MK family protein, wherein said gene is ligated to (b),
  - (d) a transcription termination sequence of a methanol-inducible alcohol oxidase gene (AOX1) derived from *Pichia pastoris*,
  - (e) a selection marker gene functioning in *Escherichia coli* and methylotrophic yeast,
  - (f) a replication origin functioning in *Escherichia coli*, and
  - (g) 5' AOX1 and 3' AOX1 for the site-specific homologous recombination to a methylotrophic yeast chromosomal DNA.
- (3) The vector according to (1), wherein said MK family protein is MK protein.
  - (4) The vector according to (1), wherein said MK family protein is PTN protein.
  - (5) A transformant comprising methylotrophic yeast transformed with the vector according to any one of (1) to (4).
  - (6) The transformant according to (5), wherein said transformant is pPIC9DP-hMK/SMD1168, said vector is the one according to (3), and said methylotrophic yeast is strain SMD1168.
  - (7) The transformant according to (5), wherein said transformant is pPIC9-hPTN/GS115, said vector is the one according to (4), and said methylotrophic yeast is strain GS115.
  - (8) A method for producing an intact MK family protein, said method comprising culturing the transformant according to any one of (5) to (7) and recovering secretory expression products.
  - (9) The method according to (8), said method comprising:
    - (a) culturing the transformant according to (6),
    - (b) inducing the expression of MK protein under the conditions of 20°C and pH 3 after the proliferation at pH 4, and
    - (c) recovering secretory expression products.

In general, a secretory protein is synthesized as a precursor having at its N-terminal side a sequence comprising 20 to 30 amino acids referred to as a signal sequence (pre-sequence). Furthermore, besides this signal sequence, most of hydrolases such as proteases, hormones, growth factors, etc. have an additional portion referred to as a pro-sequence, which is adjacent to the mature portion. The

reported analytical results on the function of this pro-sequence include that it is essential for the correct formation of disulfide bonds of the mature protein (Weissman, J. S. & Kim, P. S.: *Cell*, 71: 841-851, 1992), that it is involved in membrane permeability of the mature protein (Wiren, K. M. et al.: *J. Biol. Chem.*, 263: 19771-19777, 1988), and that it interacts with the mature protein to stimulate the formation of active correct conformation thereof (Winther, J. L. & Sorensen, P.: *Proc. Natl. Acad. Sci. USA.*, 88: 9330-9334, 1991). However, other functions still remain to be elucidated. It has been reported that replacing the signal sequence with other signal sequence or prepro-sequence remarkably decreases the efficiency of the removal of signal peptide and glycosylation (Cramer, J. H. et al.: *Mol. Cell Biol.*, 7: 121, 1987). Herein, the prepro-sequence is also referred to as "signal sequence". The "signal sequence" refers to not only an amino acid sequence but also a nucleotide sequence of a cDNA encoding the amino acid sequence.

Among secretory proteins of yeast, signal sequence structures of genes have been elucidated for invertase (SUC2), acidic phosphatase (PHO5 and PHO3),  $\alpha$ -galactosidase (MEL1),  $\alpha$ -factor (MFA1 and MFA2), a-factor (MFA1 and MFA2), double-stranded RNA killer toxin (KILM1), killer toxin of linear DNA plasmid (KIL97 and KIL28), etc. MFA1, MFA2, KILM1, and KIL97 have prepro-structures. Signal sequence of MFA1, which is most frequently used in secretory production of foreign proteins, comprises 85 to 89 amino acid residues (J. Kurjian & I. Herskowitz: *Cell*, 36: 933, 1982).

An expression vector used to markedly elevate secretory production of a foreign protein, fundamentally comprises cDNA encoding the protein region or a gene containing no intron immediately downstream of the secretion signal, which is inserted between a promoter and terminator functioning in yeast. A signal carried by the gene for a secretory protein unique to yeast or a secretion signal derived from other than yeast is used as the secretion signal.

Expression vectors used for secretory production of an MK family protein of this invention can be constructed by the standard method described in "J. Sambrook, E. F. Fritsch, and T. Maniatis: *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor

Laboratory Press, Cold Spring Harbor, New York, USA, 1989". An expression vector of appropriate methylotrophic yeast can be used. Vectors suitable for implementing this invention are those functioning in preferably the genus *Pichia* and most preferably *Pichia pastoris* GS115 (accession number: NRRL Y-15851). For example, the expression vector pPIC9 (Phillips Petroleum Co.) shown in Fig. 5 is used as one of the preferred expression vectors in this invention. The vector is a shuttle vector carrying, as the selection marker, genes required for selection in *Escherichia coli* and *Pichia pastoris*, namely, ampicillin resistance gene and *Pichia* histidinol dehydrogenase gene (HIS4). In addition, this expression vector comprises the following elements:

- replication origin functioning in *Escherichia coli* (ColE1),
- promoter of *Pichia* alcohol oxidase for expression of a foreign gene (5' AOX1),
- DNA encoding *Saccharomyces cerevisiae*  $\alpha$ 1-factor secretion signal (S),
- transcription termination sequence of AOX1 gene (3' AOX1-TT), and
- 3' AOX1 involved, together with 5' AOX1, in the site-specific integration into *Pichia pastoris* chromosomal DNA.

The two insertion site in an  $\alpha$ 1-factor signal sequence for foreign gene have been reported: (1) at the C-terminal side of Lys 85 and (2) at the C-terminal side of Ala 89 (Fumio Hishinuma: Kagaku To Seibutsu, 26: 568-576, 1988). This invention is characterized in that, an  $\alpha$ 1-factor signal sequence of *Saccharomyces cerevisiae* as shown in Fig. 3 is used in place of the signal sequence of an MK family protein itself. It is preferable to insert the gene encoding a mature MK protein immediately downstream of the spacer sequence (Glu-Ala-Glu-Ala) following the final Lys-Arg sequence of the prepro-sequence of  $\alpha$ 1-factor (i.e. the insertion site (2) described above) or at the EcoRI site located downstream of it. The gene encoding a mature PTN protein is preferably inserted immediately downstream of the final Lys-Arg sequence.

The structural genes for MK family proteins are known. The human MK gene encodes a signal peptide with 22 amino acids stretching from Met (ATG at 1 to 3) to Ala (GCC at 67 to 69) followed by a mature

protein with 121 amino acids from Lys (AAA at 67 to 69) to Asp (GAC at 427 to 429) (cf. the nucleic acid sequence of SEQ ID NO: 1 and the amino acid sequence of SEQ ID NO: 2). The gene for PTN protein encodes a signal peptide with 32 amino acids from Met (ATG at 1 to 3) to Ala (GCA at 94 to 96) followed by a mature protein with 136 amino acids from Gly (AAA at 97 to 99) to Asp (GAT at 502 to 504) (cf. the nucleic acid sequence of SEQ ID NO: 6 and the amino acid sequence of SEQ ID NO: 7). MK family proteins of the present invention include not only proteins comprising the amino acid sequence identical to the natural amino acid sequence but also mutants having the activity functionally equivalent to MK protein and comprising the amino acid sequence in which one or more amino acids are substituted, deleted, added, and/or inserted. Furthermore, intact MK protein of this invention means a protein that comprises at least the amino acid sequence of a mature protein of MK protein, which is not glycosylated. Activity of an MK family protein can be evaluated, as described in examples, for example, by the cell proliferation-promoting activity for the fibroblast strain NIH3T3 derived from a fetal Swiss mouse. In addition, those skilled in the art can modify the MK or PTN gene sequences without damaging biological functions of the corresponding protein.

A gene encoding an MK family protein can be amplified by PCR using a set of sense and antisense PCR primers suitable for amplification of the gene (in the case of MK gene, SEQ ID NOS: 3 or 5 and 4, and in the case of PTN gene, SEQ ID NOS: 8 and 9) with the gene of the protein as a template. In this case, primers are designed so as to include the appropriate restriction enzyme recognition site which is contained in the expression vector. Then, the gene is inserted into the appropriate restriction enzyme cleavage site of an expression vector. *Escherichia coli* strain HB101 or XL1-Blue MRF' is transformed with the expression vector containing the gene of a mature MK family protein. Several transformed *Escherichia coli* clones are selected, and PCR is performed with the expression vector contained in these clones as the template using appropriate primers to confirm that the inserted gene is in the correct orientation. The nucleotide sequences of the MK gene and region near the insertion site of this expression vector are determined to confirm no error in the sequence.

Yeasts used as a host include any of appropriate methylotrophic yeasts. The methylotrophic yeast includes strains capable of proliferation in the presence of methanol, such as those belonging to genera *Hansenula*, *Candida*, *Kloeckera*, *Pichia*, *Saccharomyces*, and *Rhodotorula*. Methylotrophic yeast belonging to genus *Pichia* such as an auxotrophic *Pichia pastoris* strain GS115 (NRRL Y-15851), etc. is preferable. When methylotrophic yeast with its protease activity reduced such as SMD1168 (Phillips Petroleum Company) is used as the host, the decomposition of expression product by protease can be suppressed. Known methods such as protoplast method (Hinen, A. et al.: Proc. Natl. Acad. Sci. USA, 75: 1929, 1978), lithium method (Ito, H. et al.: J. Bacteriol., 153: 163, 1983), or electroporation method [D.M. Becker and L. Guarente, "Method in Enzymology" ed. by C. Guthrie, G. Fink, Vol. 194, p. 182, Academic Press, New York (1991)], and the like can be used as the transformation method for transferring the expression vector containing the gene encoding an MK family protein. For example, when electroporation method is used, the protocol of Invitrogen (e.g., pPICZA $\alpha$ , B, C, Version A, 160410, 25-0150) can be employed.

Methylotrophic yeast thus transformed can be isolated by selecting auxotrophic yeast in growth medium containing no nutrient (depending on the auxotrophy of the yeast) and then those with a novel phenotype ("capability to utilize methanol +/-, Mut +/-"). When the transformant contains a drug resistance gene, the transformant can be selected by culturing in the presence of antibiotic that is toxic to yeast. However, isolation methods are not limited to them.

Transformed methylotrophic yeast thus isolated is cultured by suitable culturing techniques such as shaking culture in a flask, high density culture, etc. An MK family protein is expressed by the method suitable for the expression regulation region contained in the vector. For example, in pPIC9, in which the promoter of alcohol oxidase is the expression regulation region, the expression of a gene under the regulation of the promoter can be induced by culturing transformed yeast in the presence of methanol (cf. Unexamined Published Japanese Patent Application (JP-A) Nos. Hei 7-111891 and Hei 8-228779).

Figure 6 compares by ELISA expression levels of MK protein in

the fermentor cultures of the expression strain using the signal sequence of human MK protein (pHILD4-hMK/GS115) and the expression strain containing the signal sequence of  $\alpha 1$  factor (pPIC9K-4AhMK/GS115). In the case of the expression strain containing the signal sequence of human MK protein, the expression level is 30 to 50 mg/L in the 4-day culture, while, in the case of the expression strain containing the signal sequence of  $\alpha 1$  factor, the expression level is about 200 mg/L, which rises to about 240 mg/L by addition of EDTA to the culture medium. On the other hand, in the assay of the same sample by FPLC analysis, as shown in Fig. 7, the expression level obtained by addition of EDTA is about 640 mg/L (MK protein 1 mg/mL = 1.8  $A_{280}$ ), clearly demonstrating that expression level can be increased 5-fold or more by using the signal sequence of  $\alpha 1$  factor than the case of using the signal sequence of human MK itself. After the expression strain utilizing the signal sequence of MK itself (pHILD4-hMK/GS115) is cultured, MK thus expressed is purified with SP Sepharose and heparin Sepharose and subjected to mass spectrometry by MALDI method (matrix-assisted laser desorption ionization/time-of-flight mass spectrometer). The results are shown in Fig. 11. Although the peak for intact MK is highest (13241.6), peaks of MK presumably associated with 1 to 18 sugar molecules are observed, and the total amount of these peaks greatly surpass the expression level of intact MK.

Mass spectrometry of MK protein purified from the supernatant of fermentor culture of the expression strain pPIC9K-4AhMK/GS115 showed, besides intact MK protein, proteins with deletions of 5 amino acids Tyr-Val-Glu-Phe-Lys, 7 amino acids Tyr-Val-Glu-Phe-Lys-Lys-Lys, and 12 amino acids Tyr-Val-Glu-Phe-Lys-Lys-Lys-Asp-Lys-Val-Lys-Lys, from the amino terminus, as shown in Fig. 8. In addition, amino terminal analysis of expression products detected Tyr, Lys, and Asp, and these residues match amino acids at the amino termini of proteins resulted from deletion of five and seven amino acids from the amino terminus of the expected expression product MK protein. However, no clear signal for glycosylated MK is observed. These results clearly indicate association of few sugars with MK.

It is evident that use of the signal sequence of  $\alpha 1$  factor

remarkably elevates the expression level of intact MK protein and reduces glycosylation of an MK family protein as compared with the case of using the signal sequence of MK itself.

Furthermore, FPLC measurement of the expression level of MK protein in the fermentor culture of the expression strain pPIC9DP-hMK/SMD1168 shows that, as represented in Fig. 9, the expression level reaches the maximum (360 mg/L) on the eighth day of culture. Mass spectrometry of MK protein purified from 10 ml of the culture solution on the seventh day gives, as shown in Fig. 10, a value 13241.2 (+1), approximately the same as the value 13241.3 (+1), the theoretical molecular weight of MK protein, showing no signal due to the glycosylated MK. When using SMD1168 in place of GS115 as the host and altering culture conditions, the amount of degradation products has become extremely small. As shown in Table 1, the amino acid sequence at the amino terminus of the expression product coincides with that of intact MK protein. The result of amino acid composition analysis also matches well the expected values and values found for the chemically synthesized mature MK protein (standard substance) as shown in Table 2.

When the thus-obtained intact MK protein of this invention is examined for its biological activity based on the cell proliferation-promoting activity for NIH3T3 cells, a dose-dependent increase in the number of viable cells is observed as shown in Fig. 13.

Results of circular dichroism (CD) spectrum measured to obtain the secondary structure of the intact MK protein obtained by this invention are shown in Fig. 12. Overall pattern of the spectrum resembles a spectrum of protein comprising antiparallel  $\beta$ -structures. A shoulder presumably derived from a negative peak near 215 nm is clearly observed, which is thought to indicate  $\beta$ -structures. The results of CD spectrum is well consistent with the reported results of NMR analysis for MK protein showing that almost nothing but  $\beta$ -structure (Iwasaki, W. et al.: EMBO J. 16: 6936-6946, 1997). These results indicate that the intact MK protein obtained by this invention well retains the tertiary structure of MK protein. In addition, in this invention, intact PTN protein can also be obtained in such a



high yield as 250 mg/L as shown by the HPLC elution profile in Fig. 14 and by the results of mass spectrometry in Fig. 16. Thus, this invention also provides a high level secretory expression system for intact PTN protein.

5

#### Brief Description of the Drawings

Figure 1 shows the nucleotide sequence of secretion signal portion of *Pichia* yeast acidic phosphatase. Symbol v represents the cleavage site of the corresponding signal peptide.

10

Figure 2 represents the structure of pPHIL-S1 used as the expression vector for MK protein. The vector comprises *Pichia* yeast alcohol oxidase gene promoter (5' AOX1), signal sequence (S) of *Pichia* yeast acidic phosphatase (PHO1), DNA sequence (3' AOX1-TT) for AOX1 gene transcription termination, and 3' AOX1, which is, together with  
15 5' AOX1, involved in the site-specific integration into the *Pichia* yeast chromosomal DNA, including a multicloning site immediately downstream of the signal sequence. The vector comprises the replication origin of ColE1 and that of bacteriophage f1 for its replication initiation in *E. coli*, and ampicillin resistance gene  
20 (Ampicillin) and *Pichia* yeast histidinol dehydrogenase gene (HIS4) as the selection markers.

20

Figure 3 represents the nucleotide sequence of  $\alpha 1$  factor secretion signal portion originating from *Saccharomyces cerevisiae*. "(1)" represents the cleavage site of pre-sequence, "(2)", that of  
25 prepro-sequence, and "(3)", that of dipeptidylaminopeptidase.

25

Figure 4 represents the structure of pPIC9K used as the expression vector for MK protein. The vector comprises *Pichia* yeast alcohol oxidase gene promoter (5' AOX1), signal sequence (S) of  $\alpha 1$  factor derived from *Saccharomyces cerevisiae*, DNA sequence (3' AOX1-TT) for  
30 AOX1 gene transcription termination, and 3' AOX1, which is, together with 5' AOX1, involved in the site-specific integration into *Pichia* yeast chromosomal DNA, including a multicloning site immediately downstream of the signal sequence. The vector comprises the replication origin of ColE1 as that in *E. coli*, and ampicillin resistance  
35 gene (Ampicillin), *Pichia* yeast histidinol dehydrogenase gene (HIS4), and kanamycin resistance gene (Kanamycin) as the selection markers.

30

35

Figure 5 represents the structure of pPIC9 used as the expression vector for MK protein. The vector comprises *Pichia* yeast alcohol oxidase gene promoter (5' AOX1), signal sequence (S) of  $\alpha$ 1 factor derived from *Saccharomyces cerevisiae*, DNA sequence (3' AOX1-TT) for AOX1 gene transcription termination, and 3' AOX1, which is involved, together with 5' AOX1, in the site-specific integration into *Pichia* yeast chromosomal DNA, and also includes a multicloning site immediately downstream of the signal sequence. The vector comprises the replication origin of ColE1 as that in *E. coli*, and ampicillin resistance gene (Ampicillin) and *Pichia* yeast histidinol dehydrogenase gene (HIS4) as the selection markers.

Figure 6 represents the results of ELISA assay for the expression level of MK protein in the supernatant of fermentor culture of the MK protein expression strain pPIC9K-4AhMK/GS115 (comprising an  $\alpha$ 1 factor signal sequence derived from *Saccharomyces cerevisiae*) in the presence or absence of 5 mM EDTA, and the expression level of MK protein in the supernatant of fermentor culture of the strain PHILD4-hMK/GS115 (comprising the secretion signal sequence unique to MK protein).

Figure 7 represents the results of FPLC measurement of the expression level of MK protein in the culture supernatant measured in Fig. 6.

Figure 8 shows the results of MALDI mass spectrometry of MK protein purified from the culture supernatant of the MK protein expression strain pPIC9K-4AhMK/GS115 (comprising an  $\alpha$ 1 factor signal sequence derived from *Saccharomyces cerevisiae*).

Figure 9 represents the results of FPLC measurement of the expression level of MK protein purified from the fermentor culture supernatant of the MK protein expression strain pPIC9DP-hMK/SMD1168 (comprising an  $\alpha$ 1 factor signal sequence derived from *Saccharomyces cerevisiae*).

Figure 10 shows the results of mass spectrometry of MK protein purified from the culture supernatant of the intact MK protein expression strain pPIC9DP-hMK/SMD1168 (comprising an  $\alpha$ 1 factor signal sequence originating from *Saccharomyces cerevisiae*).

Figure 11 shows the results of mass spectrometry of MK protein purified from the fermentor culture supernatant of the expression

strain pHILD4-hMK/GS115.

Figure 12 shows a CD spectrum of MK protein purified from the fermentor culture supernatant of the expression strain pPIC9DP-hMK/SMD1168.

5        Figure 13 shows the growth activity of NIH3T3 cells induced by intact MK protein.

Figure 14 shows an HPLC (PolySULFOETHYL A; Poly LC) elution profile of the fermentor culture supernatant of the strain pPIC9-hPTN/GS115.

10       Figure 15 shows the results of mass spectrometry of human PTN purified from the fermentor culture supernatant of the expression strain pHILD4MK-hPTN/GS115 comprising the secretion signal of human MK.

15       Figure 16 shows the results of mass spectrometry of human PTN purified from a fermentor culture supernatant of the expression strain pPIC9-hPTN/GS115.

20       Figure 17 shows a CD spectrum of human PTN purified from a fermentor culture supernatant of the expression strain pPIC9-hPTN/GS115. Ordinate (CD value) represents the molar ellipticity  $[\theta \text{ (deg} \cdot \text{cm}^2 \cdot \text{decimol}^{-1})]$ .

#### Best Mode for Carrying out the Invention

In the following, the present invention is more specifically described with reference to examples, but is not construed as being limited thereto.

25

#### Example 1. Construction of expression vector for MK protein

30       Three kinds of expression vectors for human MK protein containing secretion signal sequences were constructed. Construction of the expression vectors was carried out according to the standard method as described, for example, by J. Sambrook, E. F. Fritsch and T. Maniatis (Molecular Cloning: A Laboratory Manual, Second Edition (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA).  
(1) Expression vector comprising the secretion signal sequence unique to MK protein

35       The MK protein expression vector "pHILD4-hMK" according to Example 1 of JP-A Hei 9-95454 was used as the expression vector

comprising the secretion signal sequence unique to MK protein.

(2) Construction of expression vector comprising the secretion signal sequence of PHO1

The expression vector PHIL-S1 (PHILLIPS PETROLEUM Co.) (Fig. 2) comprising the signal sequence of PHO1 (Fig. 1) was used. The vector comprises AOX1 promoter, signal sequence of PHO1, multicloning site in the sequence, HIS4 gene and ampicillin resistance gene as the selection markers, and other sequences. PCR was performed to amplify the mature MK cDNA using cDNA encoding human MK protein (SEQ ID NO: 1) as the template, and the sense PCR primer (SEQ ID NO: 3) and antisense primer (SEQ ID NO: 4) that contained restriction enzyme EcoRI recognition site. MK cDNA was completely digested with the restriction enzyme EcoRI, and inserted into the EcoRI site of the expression vector pHILS1 which had been digested with EcoRI and dephosphorylated with phosphatase to obtain the MK protein expression vector "pHILS1-3AhMK". *Escherichia coli* strain HB101 was transformed with the expression vector. Several clones of the transformed *Escherichia coli* were selected. Using the expression vector contained in these clones as the template and a set of appropriate primers, PCR was carried out to confirm that the inserted cDNA was oriented in the right direction. Furthermore, nucleotide sequences of the MK cDNA and the region near the insertion site thereof on the expression vector were determined to confirm the absence of any errors in the nucleotide sequence. Even though normally processed from the expression vector pHILS1, the mature MK protein possesses three excessive amino acid residues at its amino terminus.

(3) Construction of expression vector comprising the  $\alpha 1$  factor secretion signal sequence (part 1)

The expression vector pPIC9K (Fig. 4) comprising a secretion factor signal sequence of the  $\alpha$ -pheromone gene (MF $\alpha$ 1) from *Saccharomyces cerevisiae* (hereafter referred to as " $\alpha 1$  factor secretion signal") (Fig. 3) was used. The expression vector further comprises the kanamycin resistance gene for the multi-copy selection by G418 in the above-described expression vector pHILS1. According to the above-described method (2), MK cDNA was inserted into the expression vector pPIC9K to obtain the MK protein expression vector

"pPIC9K-4AhMK". Even though normally processed from the expression vector, the mature MK protein possesses four excessive amino acid residues at its amino terminus.

- (4) Construction of expression vector comprising the  $\alpha 1$  factor secretion signal sequence (part 2)

The expression vector pPIC9 (Fig. 5) comprising the  $\alpha 1$  factor secretion signal was used. The mature MK cDNA to be inserted into pPIC9 was prepared in a similar way as described in (2) using a set of sense PCR primer (SEQ ID NO: 5) and antisense PCR primer (SEQ ID NO: 4).

MK cDNA thoroughly digested with restriction enzymes EcoRI and XhoI was inserted into the expression vector pPIC9 that had been digested similarly and dephosphorylated with phosphatase to obtain the MK protein expression vector "pPIC9DP-hMK".

15

Example 2. Transformation of *Pichia* yeast with MK protein expression vector

Transfer of the MK protein expression vector into *Pichia* yeast strains GS115 and SMD1168 was carried out according to the protocol for electroporation provided by Invitrogen (for example, pPICZA $\alpha$ , B, and C, Version A, 160410, 25-0150). SMD1168 is a pep4<sup>-</sup> strain with the reduced protease activity.

Four kinds of MK protein expression vectors, pHILD4-hMK, pHILS1-3AhMK, pPIC9K-4AhMK, and pPIC9DP-hMK, were thoroughly digested with the restriction enzyme SacI or BglII. After washing with distilled water and 1 M sorbitol, GS115 or SMD1168 cells at the early logarithmic growth phase were suspended in 1 M sorbitol, and the expression vector was added thereto. Using a GenePulser of Bio-Rad, electroporation was performed under the conditions of 1.5 kV, 25  $\mu$ F, and 200-400  $\Omega$ . Transformants were selected first by the non-histidine requiring growth, and, if necessary, further by the G418 resistance. Through these selections, MK protein-expressing strains, pHILD4-hMK/GS115, pHILS1-3AhMK/GS115, pPIC9K-4AhMK/GS115, or pPIC9DP-hMK/SMD1168 transformed with expression vectors pHILD4-hMK, pHILS1-3AhMK, pPIC9K-4AhMK, or pPIC9DP-hMK, respectively, were obtained.

Example 3. Culture of MK protein-expressing strains in test tube or flask

The strains were cultured for one day in a completely synthetic medium, which was developed for high cell density fermentation, containing glycerol as the carbon source. Cells were once sedimented by centrifugation, and suspended in a fresh medium containing 1% methanol to induce the expression of MK protein. Methanol (1%) was added everyday with the adjustment of pH to 5 or 3, and the expression induction was continued for 3 days.

(1) Difference in the expression level due to secretion signals

Expression levels of MK protein in test tube culture supernatants of expression strains, pHILD4-hMK/GS115, pHILS1-3AhMK/GS115, or pPIC9K-4AhMK/GS115 were examined. In the case of pHILS1-3AhMK/GS115, the expression level of MK protein was extremely low, that is, about 0.1 mg/L at most. In the case of the expression strain pHILD4-hMK/GS115, the secretion of 2 to 3 mg/L was observed for cells with the highest expression level. In contrast, in the case of the expression strain pPIC9K-4AhMK/GS115, the expression level reached 10 mg/L for cells with the highest expression level. These results clearly show that the use of the  $\alpha 1$  factor signal sequence can yield about 3 to 5 fold higher expression level of MK protein as compared with the case of using the secretion signal sequence unique to MK protein. The results are summarized as follows.

	Secretion signal	MK family	Expression level
PHILD4-hMK	MK	MK	2-3 mg/L
PHILS1-3AhMK	PHO1	MK	0.1 mg/L
pPIC9K-4AhMK	$\alpha 1$ factor	MK	10 mg/L (host: GS115)

(2) Difference in expression products due to pH at the time of expression

The expression strain pPIC9K-4AhMK/GS115 was allowed to express the protein at pH 3 and 5. Culture supernatants were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE). The multigel 10/20

(Daiichi Pure Chemicals, Co., Ltd.) was used as the gel plate. MK protein on the third day of culturing for expression was recognized as two bands on the gel; the band of smaller molecular weight was major at pH 5, while the upper band of larger molecular weight was predominant at pH 3. These results indicate a less degradation of MK protein at pH 3 than pH 5.

### (3) Difference in expression products due to host variety

Expression of MK protein was examined in the expression strains pPIC9K-4AhMK/GS115 and pPIC9DP-hMK/SMD1168. SDS-PAGE of the three-day expression culture supernatants clearly showed MK protein as two bands in the case of pPIC9K-4AhMK/GS115, while only one band was observed in the case of pPIC9DP-hMK/SMD1168. The results indicate that, in the case where SMD1168, a strain with a low protease activity, is used as the host, degradation of MK protein is kept low.

### Example 4 Culture of MK expression strains in fermentor

The expression strains PHILD4-hMK/GS115 and pPIC9K-4AhMK/GS115 were cultured in a fermentor according to the method of J. J. Clare et al. (BIO/TECHNOLOGY, Vol. 9, 455-460, 1991). In this case, pH during the cell proliferation was set at 4, and the culture temperature at the time of protein expression was at 20°C. MK protein in the culture supernatant on the fourth day of culturing for expression was assayed by ELISA (according to JP-A Hei 10-160735). In the case of PHILD4-hMK/GS115, the expression level of MK protein was 30 to 50 mg/L, while, in the case of pPIC9K-4AhMK/GS115, it was about 200 mg/L, and, furthermore, the expression level was elevated to about 240 mg/L by addition of EDTA to the medium (Fig. 6). When expression level of MK protein for the same samples was determined by FPLC analysis using a Hitrap-Heparin column (Pharmacia, 1 ml) (0 to 2 M NaCl in 50 mM Tris-HCl, pH 7.5, flow rate 1 ml/min) measuring the absorbance at 280 nm (with the chemically synthesized mature MK protein purchased from Peptide Institute as the standard substance), the expression level of PHILD4-hMK/GS115 was about 50 mg/L and that of pPIC9K-4AhMK/GS115 was about 580 mg/L, which rose to about 640 mg/L (MK protein 1 mg/mL = 1.8 A<sub>280</sub>) when EDTA was added (Fig. 7). The results indicate that, in the case of culture in a fermentor similarly as

in the case of culture in test tubes, use of  $\alpha 1$  factor signal sequence increased the expression level of MK protein about 5 to 8 fold as compared with the case where the secretion signal sequence unique to MK protein was used. In addition, from the analytical results of the culture supernatant by SDS-PAGE and Western blot, it has become evident that the main protein in the culture supernatant is the human MK protein which has been produced through secretory expression.

Example 5. Analysis of purified MK protein (part 1)

From the fermentor culture supernatant of pPIC9K-4AhMK/GS115, MK protein was purified using SP-Sepharose and heparin Sepharose.

The culture supernatant (10 ml) was collected, and diluted with an equal volume of distilled water. The solution was adjusted to pH 5 with ammonia, and applied to a STREAMLINE SP column (about 1 ml) (Pharmacia) equilibrated with 60 mM acetate buffer (pH 5.2). After adsorption, the column was washed with the buffer containing 0.5 M NaCl, and then, MK protein was eluted with the buffer containing 2 M NaCl. The eluate was dialyzed against 50 mM Tris-HCl (pH 7.5). The dialyzed sample was applied to a column of STREAMLINE Heparin (about 0.5 ml) (Pharmacia) equilibrated with the above-described buffer used for dialysis, and, after washing the column with the above-described buffer containing 0.5 M NaCl, MK protein was eluted with the above-described buffer containing 2 M NaCl. The eluate was dialyzed against distilled water to obtain the purified MK protein.

Mass spectrometry of the purified MK protein was performed by the MALDI method. A mass spectrometer used was VOYAGER ELITE (PerSeptive Biosystems). Sinapinic acid (10 mg/mL acetonitrile/water/TFA = 33/67/0.1) was used as the matrix. A dried sample was dissolved in water (30  $\mu$ L), the matrix solution (9 volumes) was added to the dissolved sample, and the resulting solution (1  $\mu$ L) was applied to sample plates for use. Calibration was carried out with ubiquitin (+1): 8565.89 (average) and myoglobin (+1): 16952.56 (average) as the standard proteins.

Amino acid sequence analysis of the amino terminus was performed by the Edman method. A protein sequencer used was PPSQ21 (Shimadzu).

In mass spectrometry of the purified MK protein, besides the



expected MK protein (mature MK protein with Tyr-Val-Glu-Phe added to its N-terminus), proteins with deletions of 5 amino acids Tyr-Val-Glu-Phe-Lys, 7 amino acids Tyr-Val-Glu-Phe-Lys-Lys-Lys, and 12 amino acids Tyr-Val-Glu-Phe-Lys-Lys-Lys-Asp-Lys-Val-Lys-Lys, from the N-terminus were observed (Fig. 8). In addition, as a result of the N-terminal analysis of the expressed MK protein, Tyr, Lys, and Asp were detected, and they coincided with the amino acids at the N-termini of proteins with deletions of the above-described five amino acids and seven amino acids from the N-terminus of the expected expression product MK protein. Furthermore, no obvious signals for glycosylated MK are observed. From these results, it is evident that MK protein thus expressed is not glycosylated.

It is obvious that, as compared with the case where the signal sequence unique to MK is employed, use of the  $\alpha 1$  factor signal sequence leads to a remarkable increase in the expression level of MK protein, and an extremely few yeast-specific glycosylation to the expression product.

#### Example 6. Analysis of the purified MK protein (part 2)

Expression strain pPIC9DP-hMK/SMD1168 obtained in Example 2 was cultured in a fermentor. Although the culture was carried out basically according to the method described in Example 4, cell proliferation using glycerol was continued until the cell density became about  $A_{600} = 100$ , and then methanol addition was initiated to simultaneously perform the cell proliferation and expression induction. Temperature and pH at the time of protein expression were set at 20°C and 3, respectively. Expression induction was continued for nine days. The expression level of MK protein measured by FPLC reached maximum (360 mg/L) on the eighth day of culture (Fig. 9). MK protein was purified from 10 ml of the culture supernatant on the seventh day using SP Sepharose and Heparin Sepharose as in Example 5. Separately, purification by FPLC using a Hitrap-Heparin column (Pharmacia, 1 ml) was also performed with elution by a linear gradient of NaCl in 50 mM Tris-HCl (pH 7.5).

The expressed MK protein and chemically synthesized mature MK protein (Peptide Institute) as the standard were compared by performing

the reduced SDS-PAGE, non-reduced SDS-PAGE, and native-PAGE, and no difference in the measured results including molecular weight was observed. Although native-PAGE was carried out according to the method of Davis, electrodes in the electrophoresis bath were reversely  
 5 connected because of the high isoelectric point of the protein of interest. Amino acid sequence at the amino terminus of the expressed MK protein matched that of mature MK protein as shown in Table 1.

Table 1

10 Analytical results of amino acid sequence at the amino terminus of expression products

Analysis cycle	Sort of amino acid	Amount of amino acid (pmol)
1	Lys	114
2	Lys	119
3	Lys	132
4	Asp	109
5	Lys	125
6	Val	137
7	Lys	123
8	Lys	121
9	Gly	94
10	Gly	94

In addition, the sample was dissolved in 100  $\mu$ L of pure water, an aliquot (50  $\mu$ L) thereof was taken in a glass test tube, to which  
 15 50  $\mu$ L of concentrated hydrochloric acid was added, and then the mixture was subjected to hydrolysis in a vacuum sealed tube at 110°C for 22 hr. The sample was evaporated to dryness, dissolved again in 75  $\mu$ L of pure water, and an aliquot (50  $\mu$ L) thereof was subjected to amino acid composition analysis by the amino acid analytical method using  
 20 Hitachi amino acid analyzer L8500. The results are shown in Table 2.

Table 2

Result of amino acid composition analysis of expression product (MK)

Amino acid	Expected	hMK (standard) (Peptide Institute)	rhMK (pPIC9DP-hMK/SMD1168)
Asx	8	7.66	7.70
Thr	10	9.51	9.59
Ser	3	2.81	2.78
Glx	11	11.22	11.37
Gly	16	16.00	16.00
Ala	10	9.96	10.03
Val	5	4.88	4.89
Cys	10	n.d.	n.d.
Met	0	0.00	0.00
Ile	2	1.85	1.93
Leu	1	0.98	1.01
Tyr	2	1.93	1.73
Phe	3	2.93	2.97
Lys	23	22.81	22.62
His	0	0.00	0.00
Arg	7	6.73	6.80
Trp	4	n.d.	n.d.
Pro	6	6.11	5.89

5 Except for Trp, which cannot be measured because of hydrolysis  
in hydrochloric acid, and Cys, the accurate value of which can hardly  
be obtained, the results show excellent match as compared with the  
theoretical values and values of amino acid residues in the chemically  
synthesized mature MK protein as the standard specimen (Peptide  
10 Institute), indicating that MK was obtained with high purity.

The value obtained in mass spectrometry was 13241.2 (+1 value),  
which is almost the same as the expected value of the molecular weight  
(theoretical value (+1) is 13241.3) (Fig. 10), and, furthermore, only  
a minute amount of degradation products were formed by altering the  
15 host from GS115 to SMD1168 and changing culture conditions as compared  
with the case of pPIC9K-4AhMK/GS115. No signal for glycosylation of  
MK was observed. For comparison, results of mass spectrometry of  
purified MK protein which was expressed by culturing the expression  
strain containing the signal sequence unique to MK (pHILD4-hMK/GS115)  
20 are shown in Fig. 11. Although the peak of intact MK is the highest  
(13241.6), peaks for MKs which are thought to be associated with 1  
to 18 sugar molecules are also observed. Peaks with mass numbers  
smaller than that of intact MK are thought to represent partially  
degraded MKs. Since intact MK protein can be obtained only in a limited  
25 ratio in expressed products, which contain lots of molecular species

different only in the number of bound sugars, it is difficult to purify an intact MK family protein from the expression products. Judging from the above-mentioned facts, the majority of the MK family protein obtained herein is unglycosylated intact mature MK protein. Its expression level is also markedly high as compared with the strains so far used. Therefore, by using this expression strain pPIC9DP-hMK/SMD1168 for the secretory production of an MK family protein, it is possible to carry out a high level secretory expression of intact MK protein.

To obtain information on the secondary structure of intact MK protein thus obtained, its CD spectrum was measured. The results are shown in Fig. 12.

#### Example 7. Biological activity assay

##### (1) Growth activity for NIH3T3 fibroblast

Cell proliferation-promoting activity was examined using the established fibroblast strain NIH3T3 derived from fetal Swiss mouse. NIH3T3 cells (2000 cells per well) were placed onto 96-well cell culture plates, and cultured for 24 hr in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). Then, the whole medium was replaced with a mixed medium consisting of DMEM supplemented with both 10% FCS and 0 to 5000 ng/mL of intact MK (protein concentration determined based on BCA method with BSA as the standard protein) and Ham's F-12 medium (1:1) plus ITS (10 mg/L human insulin, 10 mg/L human transferrin, and 10  $\mu$ g/L selenous acid), and the culturing was further continued for 2 days. Then, the WST-1 reagent was added to the culture medium, and the absorbance of each well was measured 4 hr later with a plate reader to count viable cells. As a result, it was evident that the viable cell count dose-dependently increased (Fig. 13).

#### Example 8. Construction of PTN expression vector

According to Example 1, the human PTN protein expression vector "pPIC9-hPTN", containing the  $\alpha$ 1 factor secretion signal sequence, was constructed. cDNA encoding human mature PTN protein was amplified by PCR using PTN cDNA (SEQ ID NO: 6) as a template and a set of sense PCR primer (SEQ ID NO: 8) and antisense PCR primer (SEQ ID NO: 9)

containing the restriction enzyme EcoRI recognition site.

Example 9. Transformation of *Pichia* yeast with the PTN protein expression vector

5 According to Example 2, pPIC9-hPTN was transferred to *Pichia* yeast strain GS115 to obtain the PTN protein expression strain pPIC9-hPTN/GS115.

Example 10. Expression of PTN protein

10 According to the method described in Example 4, pPIC9-hPTN/GS115 was cultured in a fermentor, and the culture supernatant (50 ml) was fractionated using a HPLC column (PolySULFOETHYL A, Poly LC). Elution was performed with 0.7 M Na<sub>2</sub>SO<sub>4</sub>-35 mM MPB (phosphate buffer, pH 2.7). Results are shown in Fig. 14. The peaks up to the retention time 30  
15 min and the peaks at 140-162 min are thought to correspond to low molecular weight compounds such as nucleic acid, and glycosylated PTN, respectively, and the peak at 179 min can be regarded as intact PTN protein. From the peak area, the expression level of intact PTN protein is calculated to be about 250 mg/L (PTN protein 1 mg/mL =  
20 1.56 A<sub>280</sub>).

Example 11. Purification of PTN

(1) Purification with STREAMLINE SP - STREAMLINE SP (Amersham/Pharmacia; 300 ml) was added to a column (5 x 100 cm), and  
25 a fluidized bed was prepared by the upward liquid flow. After the column was equilibrated with 20 mM acetate buffer (pH 5.5), a culture solution (4.9 L) diluted 2-fold with water was applied thereto. The column was washed with the upward liquid flow of the same buffer, and washed with the downward liquid flow. Then, elution was performed  
30 with 2 M sodium chloride-20 mM acetate buffer (pH 5.5) to obtain 200 ml of the eluted fractions.

(2) Purification by sulfated Cellulofine - A column was packed with 500 ml of the carrier, sulfated Cellulofine m (Seikagaku Kogyo), and equilibrated with 0.4 M sodium chloride-10 mM phosphate buffer (pH  
35 7.2). The above-described SP purification eluate (200 ml) was diluted 3-fold with water, and phosphate buffer was added to the diluted solution

to a final concentration of 10 mM. After the resulting solution was adjusted to pH 7.2 with 8 N sodium hydroxide, the solution was applied to the above-described column. After the column was washed with 0.7 M sodium chloride-10 mM phosphate buffer (pH 7.2), elution was performed with 2 M sodium chloride - 10 mM phosphate buffer, pH 7.2, to obtain 195 ml of eluted fractions.

(3) Purification by gel filtration - Two columns (1.3 x 60 cm each) of Superdex 75 pg (Amersham/Pharmacia) were connected in series, and equilibrated with 0.152 M sodium chloride. The eluted fraction (195 ml) described in (2) above was applied to the column, and elution was performed with the same solution to obtain 200 ml of eluted fractions.

(4) Ion exchange purification - After a column (1 x 25 cm) of polySULFOETHYL A (polyLC) was equilibrated with 0.6 M sodium chloride-20 mM acetate buffer (pH 5.5), the above-described gel filtration eluate (50 ml) was applied to the column, which was washed with 0.88 M sodium chloride-20 mM acetate buffer (pH 5.5). Just prior to the elution with the same buffer, the buffer was replaced by 2 M sodium chloride-20 mM acetate buffer (pH 5.5) to obtain 20 ml of concentrated eluted fractions. The same process was repeated four times. Results of purification are shown in Table 3.

Table 3  
Purification of recombinant human pleiotrophin (rhPTN)<sup>1</sup>

	Volume (ml)	rhPTN (mg) <sup>2</sup>	Purity (%) <sup>3</sup>	Yield (%)
Expanded bed	200	950	63	96
Sulfated Cellulofine	195	840	70	85
Gel filtration	200	828	74	84
PolySULFOETHYL A	80	713	90	72

1. Results of purification from 4.9 L of the culture medium of *Pichia* yeast.
2. Quantitated by HPLC.
3. Expressed as the percent absorbance at 280 nm measured by HPLC.

Example 12. Analysis of purified PTN proteinAmino terminal analysis

As shown in Table 4, the amino-terminal amino acid sequence of the purified PTN coincided with that of the standard intact PTN protein.

Table 4

Analytical results of the N-terminal amino acid sequence of expression product (PTN)

Analysis cycle	Amino acid	Amount of amino acid (pmol)
1	Gly	95
2	Lys	111
3	Lys	111
4	Glu	96
5	Lys	112
6	Pro	75
7	Glu	58
8	Lys	74
9	Lys	86
10	Val	67

Amino acid composition

As shown in Table 5, in the amino acid composition of the purified PTN, the expected values were almost identical to the experimental values. In this case, no degradation of cysteine due to acid hydrolysis was observed.

Table 5

Analytical results of amino acid composition of expression product (PTN)

Amino acid	Expected	rhPTN (pPIC9-hPTN/GS115)
Asx	7	6.40
Thr	12	11.25
Ser	6	5.37
Glx	20	19.79
Gly	12	11.74
Ala	7	6.83
Val	4	3.89
Cys	10	9.67
Met	2	1.85
Ile	2	1.89
Leu	7	6.78
Tyr	1	0.97
Phe	2	1.95
Lys	28	28.21
Trp	4	-
His	1	1.08
Arg	5	4.63
Pro	6	5.90

## 5 Mass spectrometry

Results of mass spectrometrical analysis by the MALDI method of purified pleiotrophin which was expressed using a human midkine secretion signal are shown in Fig. 15. In this case, besides intact pleiotrophin (15305 (+1)), pleiotrophin with Gly at the amino terminus deleted (15247 (+1)), and a large amount of the two glycosylated pleiotrophins are observed (for example, 15410.9 (+1) and 15468.3 (+1)). In contrast to this, as shown in Fig. 16, the pleiotrophin expressed by the expression strain pPIC9-hPTN/GS115 has a main peak (15302.9 (+1)), which is almost the same as the theoretical value of intact pleiotrophin (15303.8 (+1)). The peak at 15510.5 (+1) represents pleiotrophin associated with matrix molecules. Therefore, this expression strain pPIC9-hPTN/GS115 is thought to be suitably used for the secretory production of PTN protein.

## 20 CD spectrum

A purified PTN (6.1 mg/mL) was diluted with saline to a concentration of 0.203 mg/mL, and subjected to the CD spectrometrical analysis using the JASCO J-500A (temperature, room temperature (about 24°C); wave length range, 200 to 250 nm; cell length, 1 mm; number



of integration, 8). As shown in Fig. 17, a negative Cotton effect was observed around 215 nm to indicate the presence of  $\beta$  structure, clearly demonstrating structural similarity to the human midkine. In the analysis of secondary structure (Y. H. Chen, et al., Biochemistry, 11, 4120-4131 (1972)), it was found that the ratio of  $\alpha$  helix: $\beta$  helix:random structure was 1:41:58.

#### Industrial Applicability

According to the present invention, an intact MK family protein can be easily and economically prepared by genetic engineering techniques. Since the intact MK family protein according to this invention is not associated with sugars derived from yeast, it does not pose problems of antigenicity in its administration to mammals such as humans. Therefore, the intact MK family protein of this invention is useful as drug raw materials. Furthermore, since the intact MK family protein according to this invention retains the expected biological activities, it can be said that the protein is of high quality as drug raw materials or research materials for the structural and functional studies on an MK family protein.

## CLAIMS

1. A vector for secretory expression of an intact MK family protein by methylotrophic yeast, said vector comprising a gene encoding a mature MK family protein ligated to a signal sequence of  $\alpha 1$  factor derived from *Saccharomyces cerevisiae*.
2. The vector according to claim 1 comprising components (a) to (g) below:
  - (a) a promoter sequence of a methanol-inducible alcohol oxidase gene (AOX1) derived from *Pichia pastoris*,
  - (b) a signal sequence of  $\alpha 1$  factor derived from *Saccharomyces cerevisiae*,
  - (c) a gene encoding a mature MK family protein, wherein said gene is ligated to (b),
  - (d) a transcription termination sequence of a methanol-inducible alcohol oxidase gene (AOX1) derived from *Pichia pastoris*,
  - (e) a selection marker gene functioning in *Escherichia coli* and methylotrophic yeast,
  - (f) a replication origin functioning in *Escherichia coli*, and
  - (g) 5' AOX1 and 3' AOX1 for the site-specific homologous recombination to a methylotrophic yeast chromosomal DNA.
3. The vector according to claim 1, wherein said MK family protein is MK protein.
4. The vector according to claim 1, wherein said MK family protein is PTN protein.
5. A transformant comprising methylotrophic yeast transformed with the vector according to any one of claims 1 to 4.
6. The transformant according to claim 5, wherein said transformant is pPIC9DP-hMK/SMD1168, said vector is the one according to claim 3, and said methylotrophic yeast is strain SMD1168.
7. The transformant according to claim 5, wherein said transformant is pPIC9-hPTN/GS115, said vector is the one according to claim 4, and methylotrophic yeast is strain GS115.
8. A method for producing an intact MK family protein, said method comprising culturing the transformant according to any one of claims 5 to 7 and recovering secretory expression products.
9. The method according to claim 8, said method comprising:

- (a) culturing the transformant according to claim 6,
- (b) inducing the expression of MK protein under the conditions of 20°C and pH 3 after the proliferation at pH 4, and
- (c) recovering secretory expression products.

# DECLARATION (37 CFR 1.63) AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name; and

I believe that I am the original, first, and sole inventor (if only one name is listed below), or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **High Level Secretary Expression System Of Intact MK Family Protein**, specification for which

☐ is attached hereto.

☒ was filed February 12, 2001, Serial No. 09/762,842.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code §119 and/or §365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Application Serial No.	Country	Filing Date	Priority Claimed
10/236621	JP	10 August 1998	Yes
11/84583	JP	26 March 1999	Yes
PCT/JP99/04332	PCT	10 August 1999	Yes

I hereby claim priority benefits under Title 35, United States Code §119 of any provisional application(s) for patent listed below:

Application Serial No.	Filing Date	Priority Claimed
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I hereby claim the benefit under Title 35, United States Code, §120 and/or §365 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (Patented, Pending, Abandoned)
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following persons registered to practice before the Patent and Trademark Office as my attorneys with full power of substitution and revocation to prosecute this application and all divisions and continuations thereof and to transact all business in the Patent and Trademark Office connected therewith: David R. Saliwanchik, Reg. No. 31,794; Jeff Lloyd, Reg. No. 35,589; Doran R. Pace, Reg. No. 38,261; Christine Q. McLeod, Reg. No. 36,213; Jay M. Sanders, Reg. No. 39,355; James S. Parker, Reg. No. 40,119; Frank C. Eisenschenk, Reg. No. 45,332; Jean Kyle, Reg. No. 36,987; Seth M. Blum, Reg. No. 45,489; Glenn P. Ladwig, Reg. No. 46,853.

I request that all correspondence be sent to:

David R. Saliwanchik  
2421 N.W. 41st Street, Suite A-1  
Gainesville, FL 32606-6669

I further request that all telephone communications be directed to:

David R. Saliwanchik  
352-375-8100

1-00

Name of First or Sole Inventor Akira Murasugi  
Residence Tokyo, Japan JPX Citizenship Japanese  
Post Office Address c/o Meiji Milk Products Co., LTD., Nutritional Foods Development Dept.  
26-11, Midori 1-chome, Sumida-ku, Tokyo 130-8502 Japan  
Akira Murasugi Date April 11, 2001  
Signature of First or Sole Inventor

\*\*\*\*\*

Name of Second Joint Inventor Yukio Asami 2-00  
Residence Kanagawa, Japan JPX Citizenship Japanese  
Post Office Address c/o Meiji Milk Products Co., LTD., Nutrition Science Institute  
540, Naruda, Odawara-shi, Kanagawa 250-0862 Japan  
Yukio Asami Date April 6 2001  
Signature of Second Joint Inventor

\*\*\*\*\*

Name of Third Joint Inventor Isao Kido 3-00  
Residence Kanagawa, Japan JPX Citizenship Japanese  
Post Office Address c/o Meiji Milk Products Co., LTD., Pharmaceuticals Development Dept.  
540, Naruda, Odawara-shi, Kanagawa 250-0862 Japan  
Kido Isao Date April 9 2001  
Signature of Third Joint Inventor

\*\*\*\*\*

4-00

Name of Fourth Joint Inventor Hideshi Kumai  
Residence Kanagawa, Japan JPX Citizenship Japanese  
Post Office Address c/o Meiji Milk Products Co., LTD., Related Companies Administration Dept.  
540, Naruda, Odawara-shi, Kanagawa 250-0862 Japan  
Hideshi Kumai Date April 9 2001  
Signature of Fourth Joint Inventor

## SEQUENCE LISTING

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Asami, Yukio

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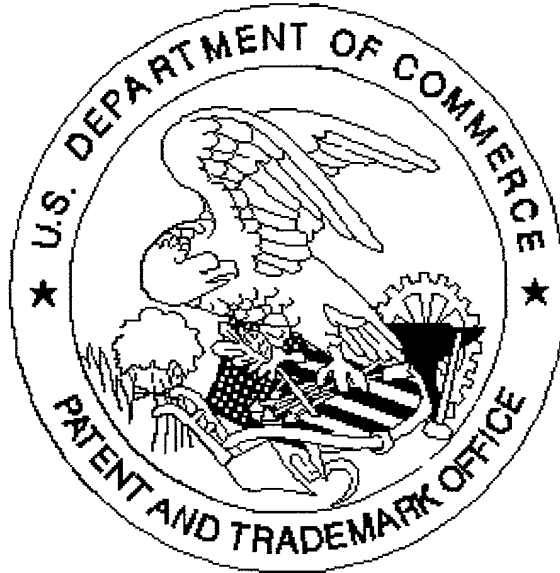
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